

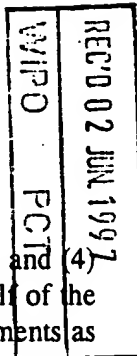


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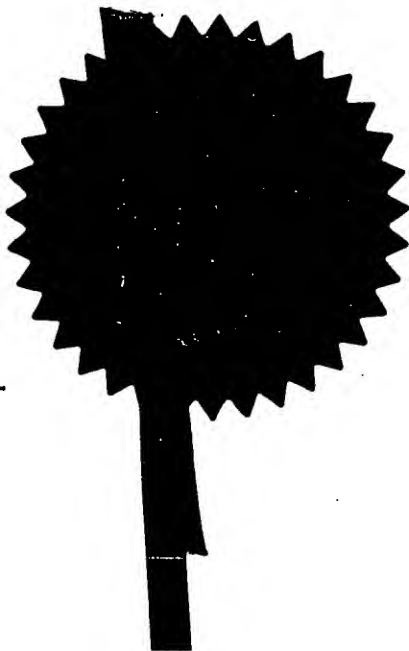
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Substances and Their Therapeutic Use

Field of the Invention

5 The present invention relates to substances and their
therapeutic use, and in particular to the identification of
regions of p21^{WAF1} that binds to cyclin dependent kinases
and/or cyclin D1, and to substances, fragments and mimetics
10 based on this region. The present invention also relates
to pharmaceutical compositions comprising these molecules
and their use in therapeutic applications for treating
hyperproliferative disorders, such as cancer and psoriasis,
and compositions comprising these molecules and their use
in applications relating to growth in eukaryotic cells.

15

Background of the Invention

The tumour suppressor function of p53 is linked to a
DNA-damage inducible cell cycle checkpoint pathway (Kastan
20 et al., 1991), in which p53 can induce either growth arrest
(Agarwal et al., 1995) or apoptosis (Clarke et al., 1993;
Lowe et al., 1993; Merritt et al., 1994) in the damaged
cells. The biochemical activity of p53 most tightly
associated with tumour suppression and growth arrest
25 involves an ionising radiation-dependent activation of
sequence-specific transcriptional activity (Kastan et al.,
1991; Lu and Lane, 1993; Pietenpol, et al., 1994). p53
induces the transcription of a number of genes, the
products of which play a direct role in mediating growth
30 arrest. These p53-inducible negative regulators of cell
proliferation include: the cyclin dependent kinase
inhibitor (CKI), p21^{WAF1} (El-Deiry et al., 1993; Harper et
al., 1993; Xiong et al., 1993; Gu et al., 1993); an
apoptosis promoting protein, Bax (Miyashita and Reed,
35 1995); the insulin growth factor binding protein IGF-BP3
(Buckbinder et al., 1995); and Gadd45 (Kastan et al.,
1992), a potent inhibitor of cell proliferation with an as
yet unclear biochemical function (Kearsey et al., 1995).

A common event in the development of human neoplasia is the inactivation of a DNA damage-inducible cell cycle checkpoint pathway regulated by p53 (Hollstein et al., 1991; Lane, 1992; Agrawal et al., 1995). A variety of mechanisms can lead to the functional inactivation of the p53 pathway, including: missense mutations within, or deletions of the p53 gene, inactivation of wild type p53 protein function by interaction with the oncogenic cellular protein mdm-2 (Momand et al., 1992), or the inability to induce downstream effector molecules, such as p21^{WAF1} (Deng et al., 1995; Waldman et al., 1995).

Our growing knowledge of the molecular mechanisms underlying the transformation of mammalian cells offers the opportunity to create rationally designed inhibitors of specific biochemical processes essential to uncontrolled cell proliferation or cancer. Recent developments have shown that the reactivation of the p53 pathway in some human tumours could in theory be achieved by: (i) activating the biochemical function of mutant p53 protein (Halazonetis and Kandil, 1993; Hupp et al., 1993), possibly using small peptides as leads for drug design (Hupp et al., 1995); (ii) disrupting the interaction of the oncogene mdm-2 and wild type p53 through the use of peptide-mimetic inhibitors of complex formation (Picksley et al., 1994); (iii) restoring or mimicking the function of the downstream effector molecule p21^{WAF1}, which on its own is capable of mediating growth suppression (El-Deiry et al., 1993; Eastham et al., 1995).

p21^{WAF1} is an inhibitor of both the G1 cyclin dependent protein kinases (CDKs; which control the progression from G1 into S phase) (Harper et al., 1995) and proliferating cell nuclear antigen (PCNA; an essential DNA-replication factor) (Florez-Rozas et al., 1994; Waga et al., 1994). Thus, inhibition of the function of either CDKs or PCNA provides, in theory, two distinct avenues for development

of drug discovery programmes which are based on the activity of p21^{WAF1}. The PCNA binding function of p21^{WAF1} can be mimicked by a 20-amino acid peptide derived from the C-terminal domain of p21^{WAF1} and this peptide is sufficient to partially inhibit SV40 replication in vitro (Warbrick et al., 1995).

Despite its PCNA binding role, the primary function of the p21^{WAF1} protein as a growth suppressor appears to be inhibition of the G1 cyclin-CDK complexes (Chen et al., 1995; Harper et al., 1995; Luo et al., 1995; Nakanishi et al., 1995b). The smallest portion of p21^{WAF1} reported to act as a CDK-inhibitor in vitro is the N-terminal domain, composed of residues 1-75 (Luo et al., 1995), which inhibits cyclin E-Cdk2.

Summary of the Invention

The present invention concerns (i) the elucidation of the molecular mechanism of cyclin D1-Cdk4 complex inhibition by p21^{WAF1}, and (ii) the identification of peptide mimetics of p21^{WAF1} inhibitory activity, through the examination of the binding and inhibitory properties of a series of synthetic peptides based on the amino acid sequence of p21^{WAF1}. Our studies found that two peptides derived from the N-terminal domain of p21^{WAF1} have biochemical activity; a peptide 4 (residues 46-65) forms a stable complex with Cdk4, but has no inhibitory activity, while a peptide 2 (residues 16-35) binds to cyclin D1 and inhibits Cdk4 activity with a Ki of 2 μ M.

These data define for the first time a cyclin binding site on p21^{WAF1} and suggest that one mechanism involved in the CDK inhibitory action of p21^{WAF1} employs binding to the cyclin subunit of the CDK holoenzyme. This has lead us to propose that p21^{WAF1} can inhibit Cdk4 activity allosterically through conformational changes in the

structure of cyclin D1. Furthermore, peptides based on the C-terminal sequence of p21^{WAF1} interact with both cyclin D1 and Cdk4, and are potent inhibitors of Cdk4 activity, with a peptide (peptide 10) composed of residues 141-160 having a K_i of 0.1 μ M. We show that both of the inhibitory peptides bind at physiologically relevant sites on cyclin D1 and/or Cdk4, and that they display specificity mimicking that of full length p21^{WAF1}. Importantly, the potency of the C-terminal peptide is improved by making a single amino acid substitution (D - A at position 149). We have mapped the inhibitory component of this peptide using alanine mutation analysis and show that it is distinct from the PCNA interaction domain, which also resides in the C-terminal region of the p21^{WAF1} protein.

Remarkably, a stretch of just five amino acids contains the Cdk4 inhibitory motif and a single conservative mutation at either of two hydrophobic amino acid residues completely abolishes the inhibitory activity of the peptide. These data have exciting implications for the mechanism of action of p21^{WAF1} protein and represent a starting point for a drug design programme aimed at producing synthetic molecules functioning as tumour suppressors downstream of p53.

Accordingly, in one aspect, the present invention provides a substance which has the property of inhibiting cdk4, said substance comprising:

- (i) a peptide fragment consisting of the motif xyLzF, wherein y and z are any amino acid and x is preferably R, or a derivative of said peptide fragment; or,
- (ii) a functional mimetic of said peptide fragment.

In a further aspect, the present invention provides the above substance for use in a method of medical treatment.

In a further aspect, the present invention provides the use of a substance which has the property of inhibiting cdk4 in

the preparation of a medicament for the treatment of a hyperproliferative disorder, said substance comprising:

- (i) fragment of the C-terminal portion of p21^{WAF1}, or an active portion or derivative thereof; or,
- 5 (ii) a peptide fragment including the motif xyLzF, wherein y and z are any amino acid and x is preferably R, or a derivative of said peptide fragment; or,
- (iii) a functional mimetic of (i) or (ii).

10 In a further aspect, the present invention provides a substance which has the property of binding to cdk4 for use in a method of medical treatment, said substance comprising:

- (i) a fragment of the p21^{WAF1} protein consisting of residues 46-65 of the p21^{WAF1} amino acid sequence, or an active portion or derivative thereof; or,
- 15 (ii) a functional mimetic of said fragment.

20 In a further aspect, the present invention provides the use of a substance which has the property of binding cdk4 in the preparation of a medicament for the treatment of a hyperproliferative disorder, said substance comprising:

- (i) a fragment of the p21^{WAF1} protein consisting of residues 46-65 of the p21^{WAF1} amino acid sequence, or an active portion or derivative thereof; or,
- 25 (ii) a functional mimetic of said fragment.

30 In a further aspect, the present invention provides a substance which has the properties of binding cyclin D and/or inhibiting cdk4 for use in a method of medical treatment, said substance comprising:

- (i) a fragment of the p21^{WAF1} protein consisting of residues 16-35 of the p21^{WAF1} amino acid sequence, or an active portion or derivative thereof; or,
- 35 (ii) a functional mimetic of said fragment.

In a further aspect, the present invention provides the use

of a substance which has the property of binding cyclin D1 and/or inhibiting cdk4 in the preparation of a medicament for the treatment of a hyperproliferative disorder, said substance comprising:

- 5 (i) a fragment of the p21^{WAF1} protein consisting of residues 16-35 of the p21^{WAF1} amino acid sequence, or an active portion or derivative thereof; or,
 (ii) a functional mimetic of said peptide fragment.

10 In the present invention, "an active portion" means a peptide which is less than the fragment of the p21^{WAF1} amino acid sequence, but which retains the relevant property mentioned above.

15 In the present invention, "functional mimetic" means a substance which may not contain an active portion of the p21^{WAF1} amino acid sequence and is probably not a peptide at all, but which has the relevant property mentioned above.

20 In the present invention, "a derivative" means a peptide modified by varying its amino acid sequence, eg by manipulation of the nucleic acid encoding the peptide or by altering the peptide itself. Such derivatives of the natural amino acid sequence may involve insertion,
25 addition, deletion or substitution of one or more amino acids, without fundamentally altering the essential activity of the peptides. An example of a derivative is the p21^{WAF1} mutant in which A was substituted for D at position 149 of the full length protein, this mutant having
30 enhanced cyclin D1-cdk4 inhibitory activity.

 In a further aspect, the present invention provides pharmaceutical compositions comprising one or more of the above substances in combination with a pharmaceutically
35 acceptable carrier.

 In a further aspect, the present invention relates to

compositions comprising one or more of the above substances and their use in controlling the growth of eukaryotic cells, eg as a food preservative or as an agent to promote the growth of plants.

5

In a further aspect, the present invention provides the use of the above substances in methods of designing or screening for mimetics of the substances.

10 Accordingly, the present invention provides a method of designing mimetics of p21^{WAF1} having the biological activity of cdk4 binding or inhibition, the activity of allosteric inhibition of cdk4 and/or the activity of cyclin D1 binding, said method comprising:

- 15 (i) analysing a substance having the biological activity to determine the amino acid residues essential and important for the activity to define a pharmacophore; and,
(ii) modelling the pharmacophore to design and/or screen candidate mimetics having the biological activity.

20

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or
25 expensive to synthesise or where it is unsuitable for a particular method of administration, eg peptides are unsuitable active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing is generally
30 used to avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property.
35 Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this

can be done by systematically varying the amino acid residues in the peptide, eg by substituting each residue in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

5

Once the pharmacophore has been found, its structure is modelled to according its physical properties, eg stereochemistry, bonding, size and/or charge, using data from a range of sources, eg spectroscopic techniques, X-ray
10 diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

15 In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this the design of
20 the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it
25 can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade in vivo, while retaining the biological activity of the lead compound. The mimetic or mimetics found by this approach can then be screened to see
30 whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for in vivo or clinical testing.

35 Brief Description of the Drawings

Figure 1. The Ability of Peptides from p21^{WAF1} to Interact

with Cdk4 and Cyclin D1.

Top panel: a list of the peptides 1-11 based on the sequence of p21^{WAF1}. Bottom Panel: The p21^{WAF1} peptides were bound to streptavidin-agarose beads and added to
 5 reticulocyte lysates containing either Cdk4 or cyclin D1 labelled with [³⁵S]methionine. After extensive washing bound proteins were analysed using SDS-PAGE followed by autoradiography. The bands were quantified using a Bio-Imager and Whole Band Analysis Software (Millipore).
 10 The results are representative of 3 such experiments.

Figure 2. Addition of p21^{WAF1} Based Peptides to Cyclin D1-Cdk4 Phosphorylation Assays.

Cyclin D1-Cdk4 assays were carried out in vitro using
 15 lysates from Sf9 insect cell following co-infection with Cdk4 and cyclin D1 baculovirus constructs and GST-Rb as the substrate. p21^{WAF1} peptides (Figure 1; Top panel) were added to the assays at a concentration of 17 μ M and the effect on Cdk4 activity was assessed by SDS-PAGE and
 20 autoradiography. Top panel: autoradiograph; bottom panel: quantification of the autoradiograph using bio-imaging, relative binding is expressed in terms of Cdk4 activity in the absence of peptide. The data are representative of 4 experiments.

25

Figure 3. Quantification of Peptide Inhibition.

Peptides 4, 8, 2 and 10 were added to cyclin D1-Cdk4 assays at various concentrations between 0.01-34 μ M. Top panel: a plot of activity (%) relative to Cdk4 activity measured
 30 in the absence of peptide against peptide concentration. Bottom panel: gives the K_i for each peptide. The data represent the mean of 3 experiments.

Figure 4. Interaction of Peptide with Cdk4 and Cyclin D1 in the Presence of p21^{WAF1}.

35 Top panel: The ability of p21^{WAF1} to interfere with peptide 2 (A) and peptide 10 (B & C) binding to Cdk4 and/or cyclin

D1 was determined by carrying out the peptide precipitation assay from reticulocyte lysates in the presence of 0, 0.5, 2 μ g of p21^{WAF1}. Bottom panel: to determine if the inhibition of Cdk4 by p21^{WAF1} could be relieved by the addition of a peptide 10 mutant, the R to A mutant (residue 15 of peptide 10) that was no longer an efficient inhibitor but still displayed partial binding activity, increasing concentrations of peptide (1, 5, 17 & 34 μ M) were added to cyclin D1-Cdk4 GST-Rb phosphorylation assay in the presence of a fixed concentration of p21^{WAF1} (50 μ M). Panel A, shows the R to A peptide 10 mutant, and panel B uses peptide 6 (Figure 1) as a control.

Figure 5. Peptides 2 or 10 are Not Substrates for Cyclin D1-Cdk4.

Top panel: phosphorylation assays using peptides 2, 4 & 10. Bottom panel: phosphorylation of p21^{WAF1} and GST-Rb were carried out as described in Experimental Procedures. Lane 1, 0.5 μ g p21^{WAF1} plus PKC; lane 2, cyclin D1- Cdk4 plus GST-Rb; lane 3, cyclin D1-Cdk4 plus GST-Rb and 0.5 μ g p21^{WAF1}; lane 4 cyclin D1-Cdk4 plus 0.5 μ g p21^{WAF1}.

Figure 6. Cyclin B-Cdc2 Assays.

To determine if peptides 2 and 10 could inhibit cyclin B-Cdc2 kinase activity assays were performed using Sf9 cell lysates which were co-expressing human cyclin B and Cdc2. The conditions were identical to those described in the Experimental Procedures for cyclin D1-Cdk4 except that histone H1 (0.5 μ g/assay) was used as the substrate. Cyclin D1-Cdk4 (panel A) and cyclin B-Cdc2 (panel B) were assayed in the presence of increasing concentrations of peptide 2 (0.25, 3, 10 and 40 μ M) and peptide 10 (0.1, 0.5, 5, 20 μ M).

Figure 7. Size Scan of Peptide 10.

Top panel: shows the sequences of a series of peptides based on peptide 10 designed to find the minimal inhibitory

domain. The boxed residues represent the minimal inhibitory domain. Bottom panel: the peptides were added to cyclin D1-Cdk4 assays and analysed by SDS-PAGE and autoradiography.

5

Figure 8. Alanine Scan Mutations of Peptide 10.

In order to pin point residues that were critical for the inhibition of Cdk4 by peptide 10 a series of point mutations were constructed in which each residue was sequentially changed to alanine. The peptides were added to cyclin D1-Cdk4 assays and the results were analysed by SDS-PAGE and autoradiography (top panel) then quantified using a Bio-Imager (bottom panel). The results are expressed relative to Cdk4 activity in the absence of peptide and are representative of 3 experiments.

15

Figure 9. Comparison of Inhibitory Peptides with Full Length p21^{WAF1} Protein.

Top panel: concentration curves for peptide 10, D to A mutant peptide 10, a p16INK4 derived peptide (Fahraeus et al., 1996) and full length his-p21^{WAF1} were determined using the cyclin D1-Cdk4 assay analysed by SDS-PAGE, autoradiography and bio-imaging. Bottom panel: data for the K_i of each inhibitor. The results are the mean of 3 such experiments.

25

Figure 10. (A) Binding and Inhibitory Domains of p21^{WAF1}.

The hatched residues show the regions of p21^{WAF1} identified in this study as being important for cyclin D1 and Cdk4 binding, and Cdk4 inhibition in the N-terminal domain, as well as a novel inhibitory domain in the C-terminus of p21^{WAF1}. The residues found to be important for the interaction of p21^{WAF1} with PCNA (Warbrick et al., 1995) are shown in black. In addition, the smallest portion of p21^{WAF1} that was found to inhibit CDK activity in vitro (Luo et al., 1995) prior to the present study is indicated.

35

(B) Model of the Allosteric Inhibition of Cyclin D1-Cdk4 Activity by p21^{WAF1}.

5 Pathway I proposes that p21^{WAF1} binds first to Cdk4 through residues (46-65) maintaining a catalytically active complex. This stable and active complex could be related to the proposed role of p21^{WAF1} as an assembly factor which promotes the formation of active cyclin-CDK complexes (Zhang et al., 1994). The induction of p21^{WAF1} protein level in response to growth inhibitory signals would then lead to the binding of a second molecule of p21^{WAF1}, through residues 16-35, to the cyclin D1 subunit causing a change in the conformation of the cyclin which allosterically inhibits Cdk4 activity. In this model, it is not known whether the binding of the second p21^{WAF1} molecule is facilitated by the presence of the original molecule nor is it known whether the mechanism whereby cyclin D1 conformational changes are transmitted to the kinase involves rearrangement of the T-loop (Pines, 1995). Alternatively (pathway II), small peptide mimetics of p21^{WAF1} (peptide 2) could bypass the physiological requirement for two molecules of p21^{WAF1} to bind and inhibit cyclin D1-Cdk4 complex activity. This provides a basis for the design of small molecular weight compounds which would prevent aberrant cell proliferation by inhibiting key G1 cyclin-CDKs.

Experimental Procedures

Peptides

30 All peptides were synthesised by Chiron Mimotopes, Peptide Systems (Clayton, Australia). Each peptide had a Biotin-SGSG spacer at the C-terminus and a free N-terminus. The peptides were dissolved in DMSO at approximately 5 mg/ml and we then determined their concentration precisely by amino acid analysis (Smythe et al., 1988). In addition the purity of the peptides was estimated using mass spectrometry. Positive ion electrospray mass spectrometry

was performed on a triple-quadruple mass spectrometer (V. G. Quattro) in (50/50/0.1) water/acetonitrile/formic acid.

Proteins

5 Cyclins and CDKs - Cdk4 and cyclin D1, and Cdc2 and cyclin B were co-expressed in Sf9 insect cells infected with the appropriate baculovirus constructs. The cells were harvested two days after infection by low speed centrifugation and the pellet was lysed in an equal volume
10 of 10 mM Hepes, pH 7.4 containing: 10 mM NaCl, 1 mM EDTA, and 0.1 mM phenylmethane sulphonyl fluoride, 2 mM DTT and centrifuged at 14000 x g for 15 min. The supernatant was removed, aliquoted and immediately frozen in liquid nitrogen. Thawed lysate was used only once and was never
15 refrozen. Labelled Cdk4 and cyclin D1 were produced by translation in the presence of [³⁵S] methionine using a rabbit reticulocyte lysate in vitro translation kit (Promega).

20 His-tagged p21^{WAF1} - Human p21^{WAF1} was expressed in E.coli using a PET expression vector. The soluble p21^{WAF1} protein fraction was purified using a nickel chelating column, following the manufacturers instructions (Pharmacia). The eluted protein peak was dialysed against 25 mM Hepes, pH
25 7.4, containing: 0.1 mM EDTA, 1 mM benzamidine, 0.01% Triton X-100, and 0.1 mM phenylmethane sulphonyl fluoride, concentrated and applied to a Superose 12 gel-filtration column (Pharmacia) equilibrated in the above buffer. Fractions containing p21^{WAF1} were detected by Western blot
30 using the p21^{WAF1} specific monoclonal antibody Ab-1 (Oncogene Sciences), concentrated to 200 µg/ml and stored at -70°C.

35 GST-Rb - An E. coli expression construct containing the hyperphosphorylation domain of pRb (amino acids 773-924) was purified on a glutathione-Sepharose column according to the manufacturers instructions (Pharmacia).

Peptide Precipitation of Cdk4 and Cyclin D1

A 20 amino acid peptide library, that spanned the entire sequence of p21^{WAF1} (Figure 1), was screened for Cdk4/cyclin D1 interacting peptides. Peptide (1.5 μ g) was diluted in 100 μ l of PBS and incubated with 10 μ l of packed streptavidin-agarose beads (Sigma) for 1 h at room temperature. Unbound peptide was removed by extensive washing with PBS and the beads, plus bound peptide, were incubated for 1h at 4°C with reticulocyte lysate containing either Cdk4 or cyclin D1 labelled with [³⁵S] methionine. The beads were washed three times with 1.25 x PBS containing 0.2% Triton X-100 and boiled in the presence of 0.125 M Tris-HCl, pH 6.8 containing: 4% (w/v) SDS, 20% (v/v) glycerol and 200 mM DTT. The bound protein was analysed by SDS-PAGE followed by auto-radiography and quantification of the ³⁵S-labelled protein using a Bio-Imager and Whole Band Analysis Software (Millipore).

Enzyme Assays

Phosphorylation of GST-Rb - Cdk4 activity was measured using the cyclin D1-Cdk4 containing insect cell lysate described above. Extract (1 μ l) was added to a final reaction volume of 10 μ l, containing: 50 mM Hepes, pH 7.4, 10 mM MgCl₂, 2.5 mM EGTA, 1 mM DTT, 10 mM β -glycerophosphate, 1 mM NaF, 10 mM PKI, 50 μ M ATP containing [³²P] ATP (1 000 cpm/pMol) and 0.5 μ g GST-Rb. The assays were started by the addition of the GST-Rb substrate, incubated at 30°C for 10 min (the incorporation of ³²P into GST-Rb was linear over 15-20 min) and terminated by adding SDS-PAGE sample buffer and heating at 95°C for 4 min. The samples were analysed by SDS-PAGE on 12% gels followed by auto-radiography and quantification using a Bio-Imager.

Peptide phosphorylation

The biotinylated peptides (1 μ g) were incubated for 30 min at 30 °C in a final volume of 20 μ l containing: 50 mM Hepes,

pH 7.4, 10 mM MgCl₂, 2.5 mM EGTA, 1 mM DTT, 10 mM β-glycerophosphate, 1 mM NaF, 10 mM PKI, 50 μM ATP containing [³²P]ATP (6000 cpm/pMol) and either 1 μl of cyclin D1-Cdk4 insect cell lysate, 1 μl of uninfected insect cell lysate or 0.02 mU of protein kinase C plus 0.5 mM CaCl₂, 100 mg/ml phosphatidyl serine and 20 mg/ml diacylglycerol. The reactions were stopped by heating at 60°C for 5 min and streptavidin agarose beads were added (10 μl packed cell volume washed with 3 x PBS) and incubated with shaking at 40°C for 30 min. The beads were washed extensively with PBS containing 3% (v/v) Tween-20 and the incorporation of radioactivity into the peptides was determined by Cerenkov counting.

15 Results

Peptide-Binding Assay for Cyclin D1 and Cdk4

Using a series of synthetic peptides that span the entire sequence of p21^{WAF1} (Figure 1), we determined whether these peptides could mimic full length p21^{WAF1} protein by forming a stable complex with either cyclin D1 or Cdk4. If peptide-binding mimetics of p21^{WAF1} protein could be identified, then this would assist in identifying the minimal binding motif of p21^{WAF1} protein required for cyclin D1-Cdk4 holoenzyme inhibition and whether p21^{WAF1} was targeting the cyclin or the kinase subunit. This would also define a system for using small peptides to study p21^{WAF1} protein reaction mechanism and to design mimetic drugs.

30 The peptide-binding assay involved quantifying the amount of ³⁵S-labelled cyclin D1 or Cdk4 which bound specifically to biotinylated peptides that were captured on streptavidin coated agarose beads. The peptide-coated beads were added to extracts containing either ³⁵S-labelled cyclin D1 or Cdk4 translated in vitro, the beads were washed extensively to remove unbound protein, and the bound cyclin D1 or Cdk4

was quantified by SDS-PAGE followed by auto-radiography and bio-imaging. This is referred to below as a peptide precipitation assay and has been used previously to demonstrate evolutionary conservation of p21^{WAF1} binding to PCNA (Ball and Lane, 1996).

A Small Peptide Derived from Amino Acids 46-65 in the N-Terminal Domain of p21^{WAF1} Binds Directly to Cdk4

Using the peptide-precipitation assay, peptide 4 (from the N-terminal domain of p21^{WAF1}) bound specifically to Cdk4, but not to cyclin D1 (Figure 1). This interaction is physiologically important, since the CDK interacting domain of the p21^{WAF1} protein has previously been proposed to localise to the N-terminal domain of the molecule (Chen et al., 1995; Harper et al., 1995; Luo et al., 1995). More specifically, deletions (Nakanishi et al., 1995a) or mutations (Goubin and Ducommun, 1995) in the region of amino acids 45-71 compromise the ability of full length p21^{WAF1} to interact with Cdk2. Whether this loss of p21^{WAF1} binding function is due to, (i) mutation/deletion of residues directly involved in CDK binding, or (ii) mutations/deletion induced conformational alterations in p21^{WAF1} that prevent stable binding to CDK, has not been demonstrated. Here we show unequivocally that residues 46-65 are directly involved in the binding of p21^{WAF1} to Cdk4 and that alone they are capable of forming a stable complex with Cdk4, in the absence of cyclin D1. Thus, providing direct evidence that the N-terminus of p21^{WAF1} does contain a kinase binding domain.

A Small Peptide Derived from Amino Acids 16-35 in the N-Terminus of p21^{WAF1} Binds Directly to Cyclin D1

We were also able to define a second and distinct N-terminal interaction site on the p21^{WAF1} protein; in this case a region of p21^{WAF1} which is capable of binding to cyclin D1, but not to Cdk4 (Figure 1). Peptide 2 comprises amino acids 16-35 of p21^{WAF1} and lies within the E_{minimum}

region required for DNA synthesis inhibition in vivo, which is located between residues 17-71 (Nakanishi et al., 1995a). Our results might explain an apparent contradiction encountered by Nakanishi et al. (1995a) who found that N-terminal mutations in p21^{WAF1} protein which are outside the CDK interacting domain, although insufficient to prevent binding to the kinase, were sufficient to prevent p21^{WAF1} from acting as a growth suppressor when transfected into proliferating cells. Specifically, the direct peptide binding data (Figure 1) leads us to suggest that an N-terminal motif in the p21^{WAF1} protein, that mediates cyclin D1 binding, could be an essential step in the mechanism through which p21^{WAF1} protein functions as a growth suppressor.

A Novel Cyclin D1-Cdk4 Binding Motif Resides in the C-Terminus of the p21^{WAF1} Protein

The specificity of the peptide-precipitation assay in defining the domain of p21^{WAF1} protein required for binding to either the cyclin D1 or Cdk4 (Figure 1), indicated that using peptides to study potential interactions between p21^{WAF1} and cyclin-CDK complexes would prove to be very informative. We were intrigued however, by the finding that peptides from the C-terminus of the p21^{WAF1} protein (peptides 10 and 11) could form stable complexes with both Cdk4 and cyclin D1 (Figure 1), as peptide 10 is equivalent to the p21PBP peptide described by Warbrick et al. (1995) as representing the region of p21^{WAF1} which binds to the replication/repair protein PCNA. We can not rule out the possibility that endogenous cyclin or CDK present in the reticulocyte lysate could bind to the labelled human protein forming a bridge to the peptide. However, as peptide 2 and peptide 4 precipitate either cyclin D1 or Cdk4, respectively, this seems unlikely. These results suggest that the p21^{WAF1} protein may interact with both PCNA and cyclin-CDK complexes through the same binding motif. Peptide 11 however, binds to both Cdk4 and cyclin D1 but

not to PCNA (Figure 1) (Warbrick et al., 1995; Ball and Lane, 1996); uncoupling the PCNA binding site from the cyclin/CDK binding motif in the C-terminus of p21^{WAF1}.

5 Given that we had identified three distinct motifs from the p21^{WAF1} protein which bind specifically to cyclin D1 and/or Cdk4, we then examined whether they mimicked p21^{WAF1} protein by inhibiting kinase activity.

10 The Cyclin D1 Binding Peptide from the N-Terminal Domain of p21^{WAF1} and the Cyclin/CDK Binding Peptide from the C-Terminus of p21^{WAF1} Inhibits the Activity of Cdk4

In order to determine if any of the p21^{WAF1} peptides possessed Cdk4 inhibitory activity we tested,
 15 independently, their ability to prevent pRb phosphorylation during cyclin D1-Cdk4 assays in vitro (Figure 2). Peptides 2, 8, 10, and 11 inhibited cyclin D1-Cdk4 activity when added to the assay at 17 μ M, whereas buffer alone and the remaining peptides had no dramatic affect on Cdk4 activity.
 20 The cyclin D1 binding peptide (peptide 2) inhibited the kinase activity by approximately 80% and peptides 10 and 11, which bound both Cdk4 and cyclin D1, completely inhibited enzyme activity at this concentration. Thus, there is a correlation between the ability of the peptides
 25 to bind to Cdk4 and/or cyclin D1 and to inhibit Cdk4 kinase activity. However, this correlation breaks down in the case of kinase-binding peptide 4. This peptide maps to the CDK interaction site (Figure 10; Goubin and Ducommun, 1995; Nakanishi et al., 1995a) and there has been
 30 speculation that a peptide from this domain, capable of interacting with CDK, would mimic full-length p21^{WAF1} inhibitory activity, and would therefore provide a model for the design of novel molecules that could arrest cell cycle progression by inhibiting the G1 cyclin-CDKs.
 35 Although of high affinity for Cdk4, peptide 4 had no inhibitory activity when added to cyclin D1-Cdk4 assays at concentrations of up to 35 μ M. Our data from both

p21^{WAF1}-peptide binding data and inhibitory properties, therefore pinpoints two novel small domains of the p21^{WAF1} protein as potential candidates for small molecular weight mimetics; an N-terminal motif from amino acids 16-35 (peptide 2) and a C-terminal motif from amino acids 141-160 (peptide 10).

The C-Terminal p21^{WAF1} Peptide is a More Potent Inhibitor of Cdk4 Kinase Activity than the N-Terminal Cyclin D1-Binding Peptide

We carried out more detailed studies to determine the K_i for peptides 2, 8, and 10, using peptide 4 as a negative control (Figure 3). We found that peptide 10 (and peptide 11; data not shown) was a potent inhibitor of Cdk4 activity with a K_i of 0.1 μ M, peptide 2, was also a good inhibitor with a K_i of 2 μ M. Peptide 8 gave only weak inhibition and relatively high concentrations of peptide were required to approach 50% inhibition. These data support the possibility of using peptide 2 or peptide 10 to mimic the CDK inhibitory activity of the full length p21^{WAF1} protein.

p21^{WAF1} Protein and Inhibitory Peptides Compete for the Same Binding Site on Cdk4 Kinase

In order to determine if the Cdk4 inhibitory peptides, 2 and 10, were acting at sites on Cdk4 and cyclin D1 that were also employed by p21^{WAF1}, we carried out peptide precipitation assays in the presence and absence of full length purified his-p21^{WAF1} to find out if it competed with the peptides for binding (Figure 4, top panel). The data suggest that binding of p21^{WAF1} protein to cyclin D1 and Cdk4 prevents binding of both peptide 2 and peptide 10. These data are open to two interpretations, (i) the peptides could be competing for binding at the same site as p21^{WAF1}, or (ii) binding of either p21^{WAF1} or peptide could cause a conformational change in the cyclin or CDK preventing further binding. It is not clear from these experiments whether peptides 2 and 10 are acting at the

same site(s). However the difference in the peptide precipitation data, indicates that at least one of the sites is unique, as peptide 10 can precipitate both Cdk4 and cyclin D1, whereas, peptide 2 can only precipitate cyclin D1.

Data to support the hypothesis that peptide 10 and p21^{WAF1} protein compete for the same binding site, during kinase inhibition, employs the use of a peptide 10 mutant (containing a point mutation resulting in a change of R - A at residue 15 of peptide 10 which is equivalent to residue 155 of the full length protein) which loses >60% of its inhibitory activity (see below), but retains its binding function. The experiment showed that increasing concentrations of mutant peptide 10 were able to block the inhibitory activity of full length p21^{WAF1} (Figure 4, bottom panel), suggesting that peptide 10 is binding at a site(s) which blocks subsequent binding of p21^{WAF1} and is therefore functioning through a similar mechanism to the full length protein.

The Inhibitory Peptides are not Cyclin D1-Cdk4 Substrates
Unlike the p107 protein, which appears to inhibit Cdk4s ability to phosphorylate pRb by acting as an alternative substrate (Zhu et al., 1995), p21^{WAF1} has not been reported to act as a substrate for the cyclin D1-Cdk4 complexes (and we confirm these observations Figure 5). However, it is possible that by using p21^{WAF1} based peptides, instead of full length protein, we have inadvertently generated phosphorylation sites which would not normally be exposed on the surface of the protein. Thus the peptides could be acting as competitive substrates as opposed to inhibitors of catalytic activity. Both peptide 2 and peptide 10 contain a number of possible phosphorylation sites, and we have been able to demonstrate that peptide 10 is a potential substrate for a number of protein kinases (data not shown), including protein kinase C (PKC) which was used

as a control kinase (Figure 5). In fact, neither peptide 2 nor peptide 10 were substrates for cyclin D1-Cdk4 under conditions where 2.4 nMol of ^{32}P were incorporated per nMol of GST-Rb. However, under the same conditions peptide 10 was an extremely good substrate for PKC with 0.82 nMol of ^{32}P being incorporated per nMol of peptide (Figure 5). There was a low level of incorporation into peptide 2, but as this was also present in assays using lysate from uninfected insect cells it must be attributed to low levels of endogenous protein kinase(s). Thus, it appears that the peptide inhibitors are not competitive substrates, but, are acting to block catalytic activity in a mechanism similar to p21^{WAF1}.

15 The Peptides are not Efficient Inhibitors of Cyclin B-Cdc2 Kinase Activity

Harper et al. (1995) have shown that p21^{WAF1} is not a universal CDK inhibitor, but that it displays selectivity for the G1 and S-phase cyclin-CDK complexes. When they compared the ability of p21^{WAF1} to inhibit Cyclin B-Cdc2, which acts at the G2/M transition, and cyclin D2-Cdk4, which functions during G1, they found that the K_i for inhibition of cyclin B-Cdc2 was > 600-fold higher than the K_i for inhibition of cyclin D2-Cdk4 using purified recombinant proteins. We looked at the effect of adding our two cyclin D1-Cdk4 inhibitory peptides to cyclin B-Cdc2 assays at concentrations up to 20 μM and found that neither peptide 2 nor peptide 10 had a significant effect on the enzymes histone H1 kinase activity (Figure 6). Thus, the p21^{WAF1} based peptide inhibitors appear to have equivalent specificity to the full length protein with regard to cyclin B-Cdc2.

35 The Kinase Inhibitory Motif of Peptide 10 is Distinct from the PCNA Binding Site

We have shown that peptide 10 is an extremely potent inhibitor of cyclin D1-Cdk4 activity, with a K_i of 0.1 mM

which is 20-fold more potent than peptide 2, a peptide derived from the region of p21^{WAF1} previously associated with growth arrest (Chen et al., 1995; Nakanishi et al., 1995a). We have also shown that a peptide (peptide 4) which spans the CDK interaction site of p21^{WAF1} (Goubin and Ducommun, 1995; Nakanishi et al., 1995a), although capable of binding to Cdk4 to form a stable complex, has no detectable activity as a cyclin D1-Cdk4 inhibitor. Peptide 10 therefore looks like the best candidate for the development of a small peptide mimetic with high efficacy. Peptide 10 has previously been shown to form a specific high-affinity and reversible interaction with PCNA (Ball and Lane, 1996) and this peptide is sufficient to partially inhibit the function of PCNA during SV40 replication giving 50% inhibition at a concentration of approximately 7 mM (Warbrick et al., 1995). The PCNA interaction domain of p21^{WAF1} has been mapped and the important residues were found to be amino acids 144-151 (QTSMTDFY; Warbrick et al., 1995; Ball and Lane, 1996). Although the extreme C-terminal peptide (peptide 11) has amino acid residues important for binding to and inhibiting Cdk4 (see Figure 1 and 2), it cannot bind PCNA (Warbrick et al., 1995; Ball and Lane, 1996). These results indicate that the kinase inhibitory and PCNA binding motif in the C-terminus of p21^{WAF1} are distinct, but it does not rule out the possibility that an interaction between p21^{WAF1} and PCNA or cyclin/kinase may require some common amino acids. It is therefore important to identify the precise inhibitory motif within the C-terminus of p21^{WAF1} and establish if it overlaps, or is distinct from, the PCNA interaction domain. To investigate this question we took two approaches; we synthesised, (i) a series of peptides that had been shifted by 4 amino acids in either direction along peptide 10 (size scan; Figure 7), and (ii) a series of peptides based on peptide 10 where each residue was sequentially mutated to alanine (alanine scan; Figure 8). The ability of the peptides, in each of these two series, to inhibit Cdk4

activity in vitro was then determined. Using the size scan, we found that the peptide inhibition activity required amino acids 156-160, while amino acids 148-155 were dispensable (Figure 7). This uncouples the kinase inhibitory motif from the PCNA binding motif.

With the alanine scan we defined the critical residues for inhibition showing that a stretch of just 5 amino acids were essential for activity, with a single conservative point mutation at either of two hydrophobic residues completely abolishing peptide 10 inhibitory activity (Figure 8). The essential amino acids are RRLIF (amino acids 155-160) where the bold characters are essential for activity and the underlined residue contributes significantly to inhibitory activity.

When tested in the peptide precipitation assay, mutation of the first R of this motif to A (aa 155 of full length p21^{WAF1}) partially retained its ability to bind both Cdk4 and cyclin D1, whereas mutations of L or F to A significantly decreased the affinity for Cdk4 and cyclin D1, and mutations of the second R or the I had no effect on binding (data not shown). This is why the R - A mutant was used in competition assays (Figure 4). The fact that a single point mutation in either of two hydrophobic residues (the L or F residues) completely abolishes inhibitory activity, suggested that inhibition was due to a specific interaction at key hydrophobic residues. The mapping data also explains why both peptide 10 and peptide 11 are good inhibitors of cyclin D1-Cdk4 activity (Figure 2) as they both contain the inhibitory motif. Thus, it appears that the inhibitory portion of peptide 10 does not overlap with the PCNA binding site as they have no amino acid residues in common.

35

A Single Amino Acid Substitution in Peptide 10 Makes it a More Potent Inhibitor thus Approaching the Specific

Activity of Full Length p21^{WAF1} Protein

Whilst carrying out the alanine scan experiments we noticed that one of the mutant peptides (D - A at position 9 of peptide 10 or 149 of the full length protein; Figure 8) appeared to make the peptide a better inhibitor of cyclin D1-Cdk4 activity. We determined the K_i for this peptide and compared it with peptide 10, full length purified his-p21^{WAF1}, and a peptide derived from the tumour suppressor protein p16 INK4 which has recently been reported to inhibit cyclin D1-Cdk4 activity in vitro and to prevent cell cycle progression (Fahraeus et al, 1996). The D-A mutation decreases the K_i from 100 nM to 46 nM (Figure 9). Comparing this with the p16INK4-based peptide, which has a K_i of 16.3 μ M (Figure 9), we have now produced a peptide which is approximately 350-fold more active as a Cdk4 inhibitory compound. In fact, we now begin to approach the potency of p21^{WAF1} itself, which has a K_i of 11 nM in the insect cell lysate assay (Figure 9). This value is in the same range as the K_i of 40 nM for p21^{WAF1} obtained for the inhibition of cyclin D1-Cdk4 in Sf9 cell lysates by Harper et al. (1995). Compared to full length protein, the mutant peptide 10 has only a 3.5-fold lower specific activity as a kinase inhibitor in crude lysates. Why mutating the D - A in this position, which is well out side the domain shown to be essential for activity, reduces the K_i is not known. It seems likely that it involves the presentation of the inhibitory motif, rather than a direct role for this residue in inhibition, as this mutation does not appear to increase the affinity of the peptide for either Cdk4 or cyclin D1 (data not shown).

The results indicate that peptide 10 could be used as a model on which to base small peptide mimetics of p21^{WAF1} and we have provided evidence that alterations in the peptides structure or presentation of the active residues may lead to the generation of a peptide inhibitor which approaches

the potency of full length p21^{WAF1} as a cyclin D-Cdk4 inhibitor.

Discussion

5

Synthetic peptides or peptido-mimetics are proving to be useful in studying the biochemical regulation of enzymes and proteins, and also in providing models for the design of novel anti-proliferative agents targeted to the enzymatic pathways amplified or proteins activated in human tumours (Powis, 1992; Gibbs and Oliff, 1994). Peptides which have been shown to effectively target components of the cell cycle machinery include: FTI, which inhibit farnesyl protein transferase preventing the activation of Ras (Gibbs et al., 1994); Ras effector domain peptides, which can inhibit its biological function (Moodie and Wolfman, 1994; Rodriguez-Viciana et al., 1994); SH2/SH3 domain-harboursing polypeptides, which in theory should inhibit the growth of tumours with activated tyrosine kinases (Pawson and Schlessinger, 1993; Yu et al., 1994), and p16INK4-derived peptides, which inhibit cyclin D-CDK complex activity and thereby activate pRb-dependent cell cycle arrest (Fåhræus et al., 1996).

25 Inactivation of the tumour suppressor protein p53 is a common event in the development of human neoplasia (Hollstein et al., 1991). The p53 protein is a key player in an inducible cell cycle checkpoint pathway activated in response to DNA-damage and nucleotide pool perturbation (Lane, 1992; Agarwal et al., 1995). Reactivation of this pathway could therefore provide a route to the discovery of novel anti-proliferative drugs. A variety of mechanisms could lead to the functional inactivation of the p53 pathway, including the inactivation of downstream effector molecules of p53, such as the cyclin-kinase inhibitor p21WAF1 (Deng et al., 1995; Waldman et al., 1995). Recent developments have shown that reactivation of the p53

pathway in some human tumours may be possible by activating the biochemical function of the endogenous mutant p53 protein (Halazonetis and Kandil, 1993; Hupp et al., 1993), possibly using small peptides as leads for drug design (Hupp et al., 1995) or by reintroducing the wild type p53 gene using adenovirus vectors (Eastham et al., 1995). However, in general, the pharmacological restoration of biochemical function to a protein that has lost its normal activity through mutation of its amino acid sequence is more difficult than the inhibition of a biochemical function (Gibbs and Oliff, 1994). Thus, it may prove more productive to take alternative approaches to restore activity to the p53 pathway such as mimicking the inhibitory activity of the downstream effector molecule p21^{WAF1}, which can by itself mediate growth arrest primarily through its interaction with the G1 cyclin-CDKs (El-Deiry et al., 1993; Eastham et al., 1995; Harper et al., 1995).

Determining the minimal domain of p21^{WAF1} that can inhibit CDK function and whether such a domain can function in isolation with high efficiency are two important goals which must be achieved in order to determine whether p21^{WAF1} will prove to be a realistic template for use in anti-proliferative drug design research. Prior to our studies, the minimal sequence of p21^{WAF1} shown to inhibit CDK function in vitro was the N-terminal domain (residues 1-75) (Luo et al., 1995). Whilst peptides derived from this N-terminal domain have recently been shown to antagonise the ability of p21^{WAF1} to inhibit cyclin E-Cdk2 complex activity suggesting that this domain interacts with the kinase (Chen et al., 1996), no data on the direct interaction of small peptides with either cyclin or CDK has previously been presented. In addition, no evidence existed to suggest that a small peptide derived from p21^{WAF1} would in fact be biologically active as a CDK inhibitor. As the cyclin D1-Cdk4 complexes and related isoforms are

essential for progression through G1-phase, we have used a series of small synthetic peptides based on the sequence of p21^{WAF1} to, (i) determine whether Cdk4 inhibitory peptide-mimetics exist and if they are of high efficacy, and (ii) probe the mechanism by which the p21^{WAF1} protein inhibits cyclin D1-Cdk4 activity.

A Model for the Allosteric Inhibition of Cyclin D1-Cdk4 by p21^{WAF1}

Two distinct peptides from the N-terminal domain of p21^{WAF1} interacted with either Cdk4 or cyclin D1 to form stable complexes. One peptide bound to Cdk4 but did not inhibit its activity, while the second bound specifically to cyclin D1 and had potent inhibitory effects on cyclin D1-Cdk4 activity. The Cdk4 binding peptide 4 (residues 46-65) corresponded to a putative Cdk2 binding domain of p21^{WAF1} previously defined using p21^{WAF1} deletion constructs (Nakanishi et al., 1995a) and alanine mutation analysis (Goubin and Ducommun, 1995). We have established that this region of p21^{WAF1} is, in fact, directly involved in CDK binding, yet it has no Cdk4 inhibitory activity (Figures 2 and 3). These data explain why certain N-terminally deleted p21^{WAF1} constructs, which still contain the CDK binding site, fail to efficiently inhibit cell growth (Nakanishi et al., 1995a).

The second N-terminal peptide, which bound to cyclin D1, potently inhibited cyclin D1-Cdk4 activity through a novel mechanism (see below). The mechanism of p21^{WAF1} inhibition of cyclin-CDK complexes is poorly understood, as it has not been clear whether p21^{WAF1} protein inhibits by cyclin and/or kinase subunit binding. Cdk2 binds very weakly to p21^{WAF1} in the absence of cyclin, the affinity of the G1-CDKs for p21^{WAF1} being greatly increased if the CDK is associated with a cyclin (Harper et al., 1995), suggesting that cyclins play an important role in p21^{WAF1} inhibition of CDK activity. However, whether a CKI, such as p21^{WAF1} and

p27KIP1, can interact directly with cyclin is in dispute (Toyoshima and Hunter, 1994; Harper et al., 1995). A recent study however, suggested that p21^{WAF1} can interact directly with a number of cyclins in the absence of CDK (Fotedar et al., 1996). We show here that a small peptide composed of residues 16-35 (peptide 2) forms a stable complex with cyclin D1 and that this peptide alone is a potent inhibitor of Cdk4 activity, with a K_i of 2 mM. This peptide falls within the growth suppressor region (residues 17-71), described by Nakanishi et al. (1995a). This is the first time that a putative cyclin binding site on p21^{WAF1} has been identified and that a small synthetic peptide representing this domain has been shown to be sufficient to mimic the full length p21^{WAF1} protein as a CDK inhibitor.

The fact that cyclin D1-Cdk4 activity can be inhibited by interaction with the cyclin subunit alone, suggests that conformational changes in cyclin D1 can lead to the inhibition of Cdk4 catalytic activity. Thus, we propose that one mechanism by which p21^{WAF1} can affect Cdk4 activity is by allosteric inhibition mediated through conformational changes in the cyclin subunit. In addition, our results may offer an explanation of how active and inactive p21^{WAF1} containing cyclin-CDK complexes can exist and how the loading of multiple p21^{WAF1} molecules onto cyclin-CDKs can cooperatively inhibit kinase activity (Zhang et al., 1994; Harper et al., 1995).

In our model of p21^{WAF1} inhibition of cyclin D1-Cdk4 kinase function (Figure 10), we propose that one molecule of p21^{WAF1} preferentially binds first to Cdk4 through its CDK interacting site (residues 46-65) without affecting kinase catalytic activity. This is consistent with previous reports that cyclin-CDK complex associated with a single p21^{WAF1} molecule are catalytically active (Zhang et al., 1994). After a second molecule of p21^{WAF1} binds to the cyclin D1 subunit, through interaction with p21^{WAF1} residues

16-35, allosteric inhibition of kinase function is achieved. It is also possible that the rate of binding of the second molecule of p21^{WAF1} to the cyclin D1 subunit could be facilitated by the presence of the first at the kinase subunit, leading to cooperative inhibition.

Prospects for the design of small molecular mimetics of p21^{WAF1} are more viable given that the cyclin D1-binding peptide alone can inhibit kinase function, indicating that the prior presence of one p21^{WAF1} protein binding to the kinase subunit is not required for allosteric inhibition of kinase function. In addition, the amino acid residues that are conserved between p21^{WAF1} and its close relative p27KIP1 (Polyak et al., 1994; Toyoshima and Hunter, 1994) are clustered within the N-terminal domain, with the regions corresponding to peptides 2 (65% identical) and peptide 4 (50% identical) containing the majority of the conserved amino acids. This suggests that allosteric inhibition of Cdk4 activity by interaction with the cyclin D subunit may be a common mechanism employed by both p21^{WAF1} and p27KIP1.

A Novel p21^{WAF1} C-Terminal Cyclin D1-Cdk4 Inhibitory Domain
 During the course of our studies we also found that a peptide (peptide 10) from the C-terminal domain of p21^{WAF1} was a potent inhibitor of cyclin D1-Cdk4 activity in vitro. The inhibitory motif was identified and was distinct from the PCNA interacting site, which also resides in the C-terminal domain of p21^{WAF1} (Chen et al., 1995; Luo et al., 1995; Warbrick et al., 1995; Ball and Lane, 1996). Our results are in contrast to previous studies which have found that cyclin-Cdk2 inhibitory activity is confined solely to the N-terminal domain of p21^{WAF1}, when each half is expressed separately (Chen et al., 1995; Luo et al., 1995). The reasons for this discrepancy may include: (i) the use of C-terminally his-tagged p21^{WAF1} in expression vectors for purifying p21^{WAF1} constructs (Luo et al.,

1995), which may have affected the local structure at the C-terminus of p21^{WAF1}; (ii) the transfection of constructs containing only the C-terminal half of p21^{WAF1} (Chen et al., 1995; Luo et al., 1995) this may make folding into the correct native conformation difficult precluding identification of the novel inhibitory domain; (iii) by using peptides, rather than the C-terminal constructs or full length p21^{WAF1} protein, we may have exposed sites which would not be solvent exposed in native full length p21^{WAF1} protein; (iv) it is possible that there may be subtle differences in the mechanism(s) used by p21^{WAF1} to inhibit cyclin-Cdk2 complexes and cyclin D1-Cdk4. Whether, the C-terminal inhibitory motif defines a novel physiologically relevant regulatory site on p21^{WAF1} is currently being addressed. However, the potency of peptide 10 ($K_i = 0.1$ mM, only 10-fold lower than full length p21^{WAF1} protein in these assays) and its ability to completely inhibit cyclin D1-Cdk4, suggests to us that further studies on this region of full length p21^{WAF1} will be well worth pursuing.

Peptide 10 represents a potentially exciting lead for drug design as it is by far the most potent peptide inhibitor of CDK activity discovered to date, being > 150-fold better than the recently identified peptide mimetic of p16INK4 (Fahraeus et al., 1996) and 20-fold better than the N-terminal inhibitory p21^{WAF1}-derived-peptide which we have described. The fact that the residues important for inhibitory activity are confined to a stretch of just five amino acids, suggests that contact at a single interface is sufficient to produce a highly potent inhibitor of the cyclin D1-Cdk4 activity, making this a realistic template for the design of small molecules which mimic p21^{WAF1} activity.

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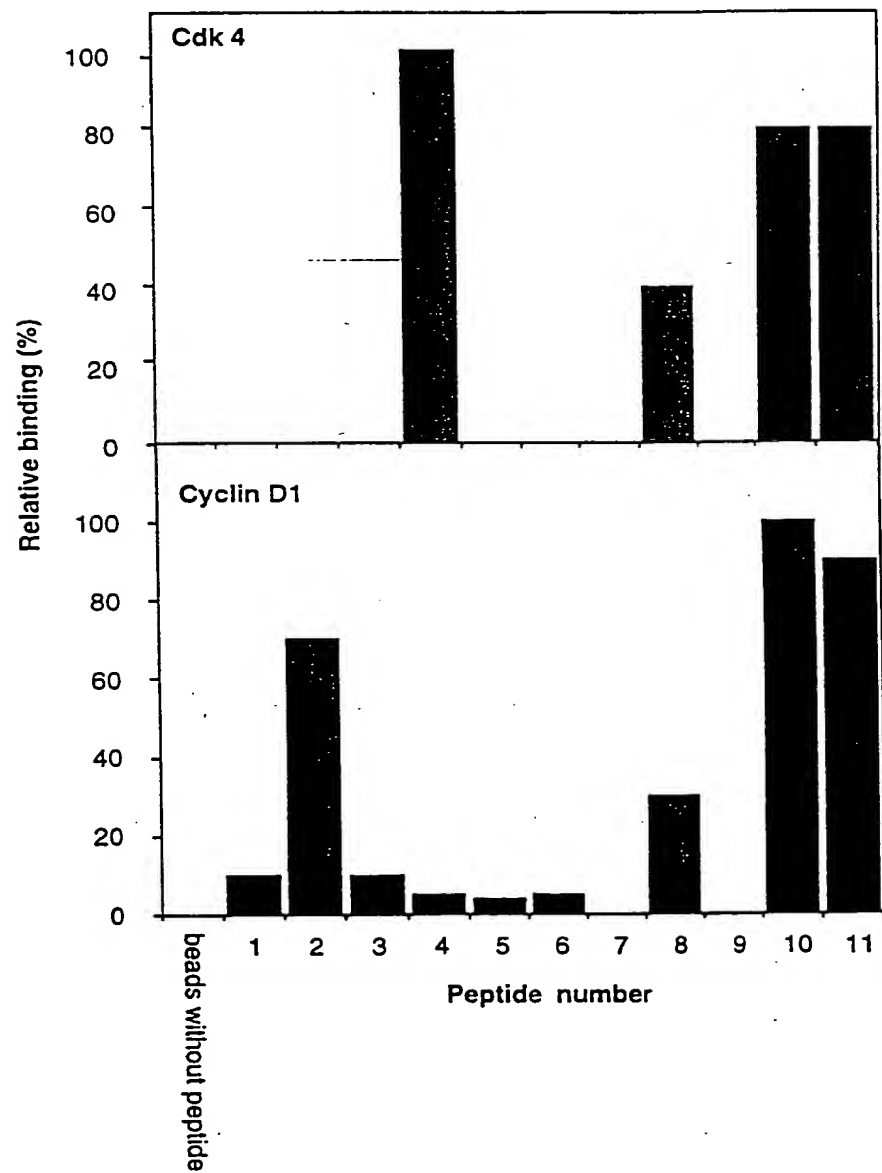
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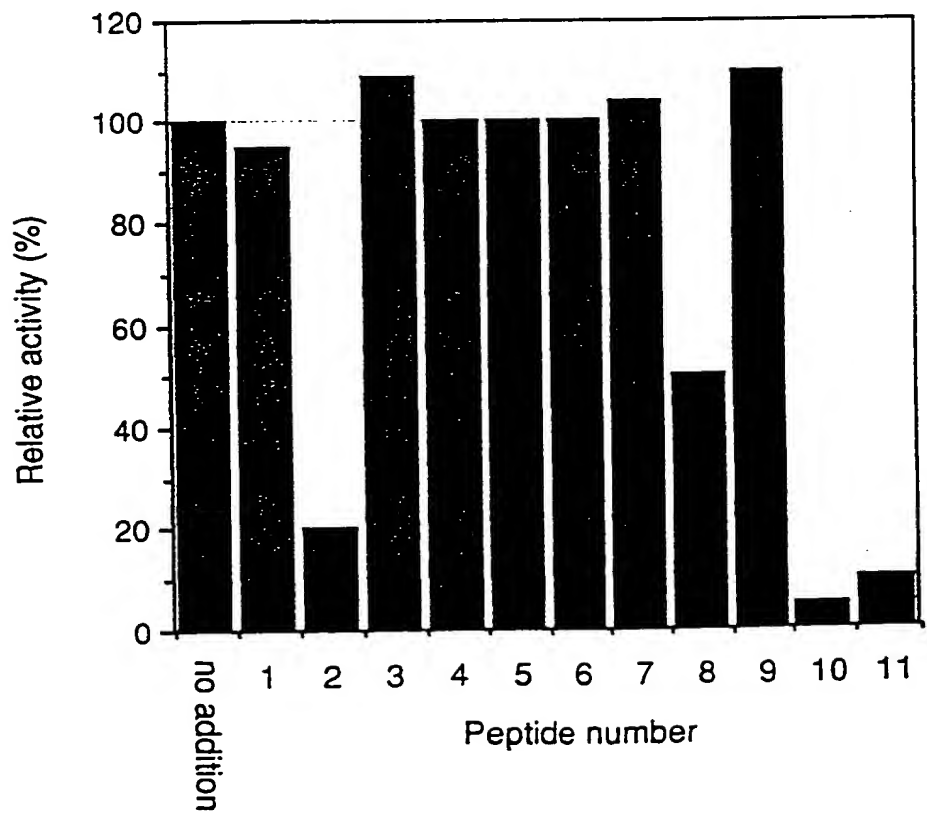
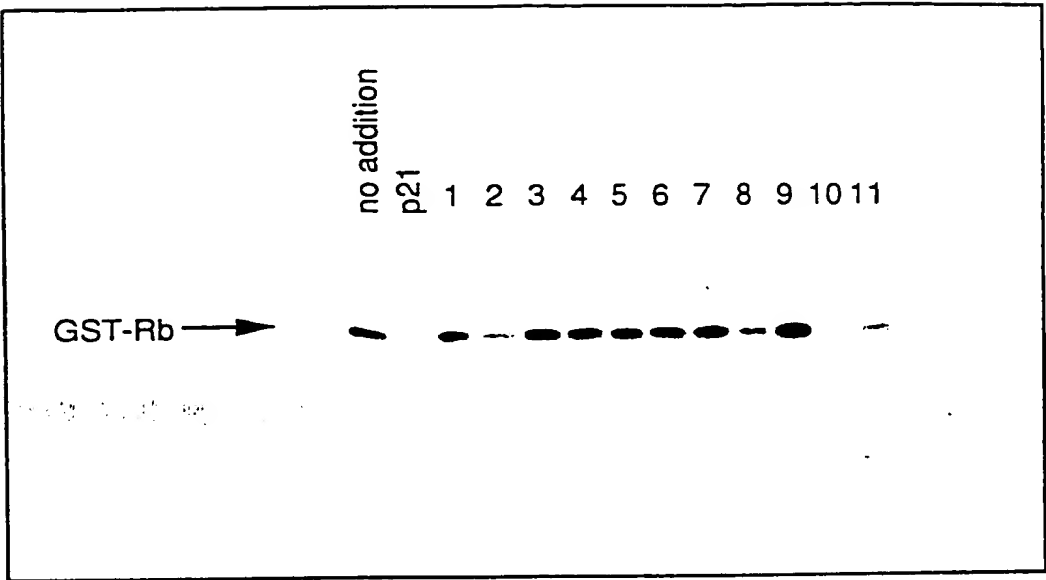
FIGURE 1

Peptide 1.	M	S	E	P	A	G	D	V	R	Q	N	P	C	G	S	K	A	C	R	R
Peptide 2.	K	A	C	R	R	L	F	G	P	V	D	S	E	Q	L	S	R	D	C	D
Peptide 3.	S	R	D	C	D	A	L	M	A	G	C	I	Q	E	A	R	E	R	W	N
Peptide 4.	R	E	R	W	N	F	D	F	V	T	E	T	P	L	E	G	D	F	A	W
Peptide 5.	G	D	F	A	W	E	R	V	R	G	L	G	L	P	K	L	Y	L	P	T
Peptide 6.	L	Y	L	P	T	G	P	R	R	G	R	D	E	L	G	G	G	R	R	P
Peptide 7.	G	G	R	R	P	G	T	S	P	A	L	L	Q	G	T	A	E	E	D	H
Peptide 8.	A	E	E	D	H	V	D	L	S	L	S	C	T	L	V	P	R	S	G	E
Peptide 9.	P	R	S	G	E	Q	A	E	G	S	P	G	G	P	G	D	S	Q	G	R
Peptide 10.	K	R	R	Q	T	S	M	T	D	F	Y	H	S	K	R	R	L	I	F	S
Peptide 11.	T	S	M	T	D	F	Y	H	S	K	R	R	L	I	F	S	K	R	K	P



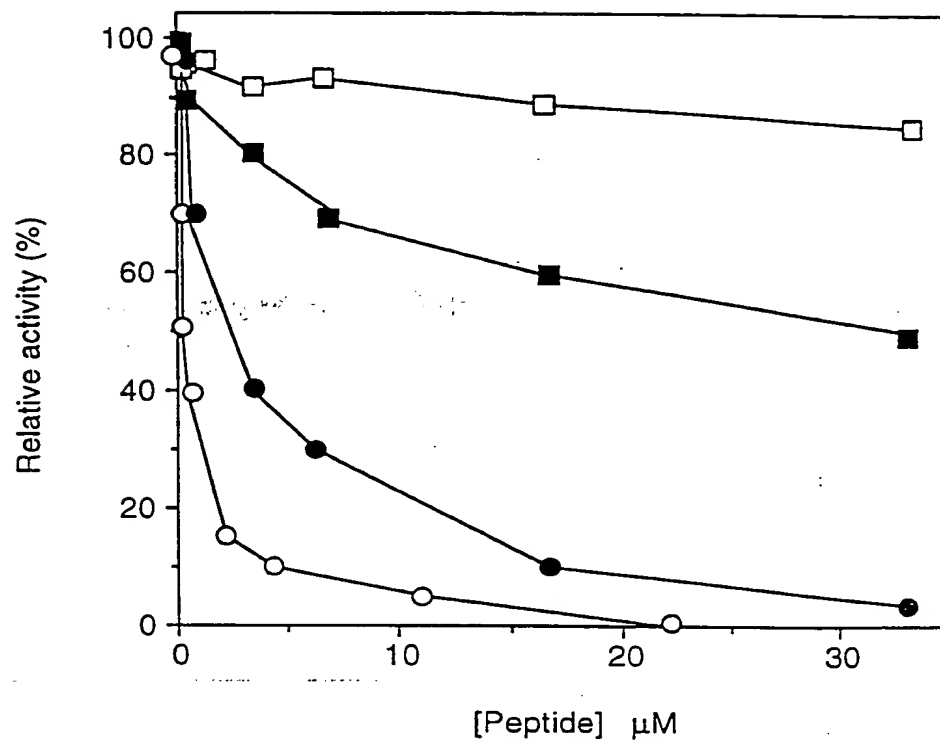
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FIGURE 2



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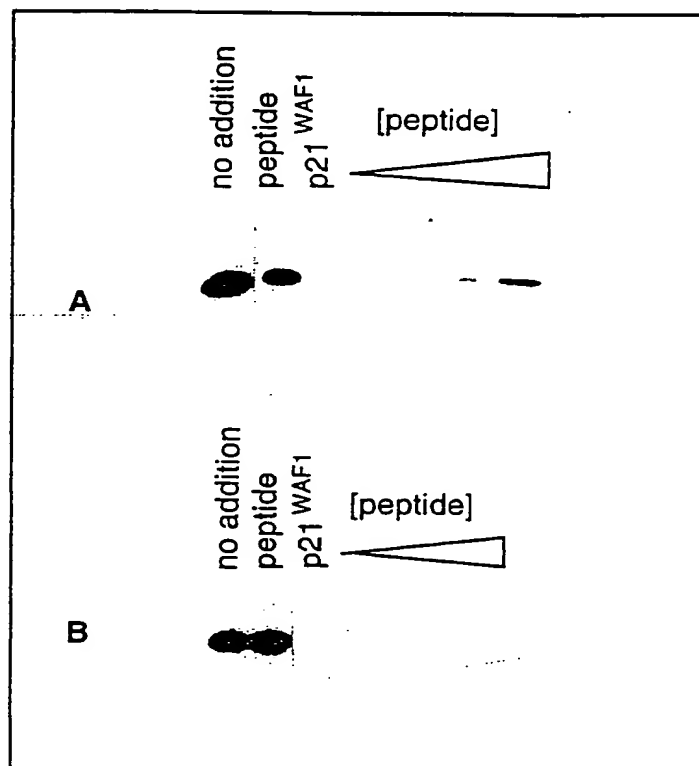
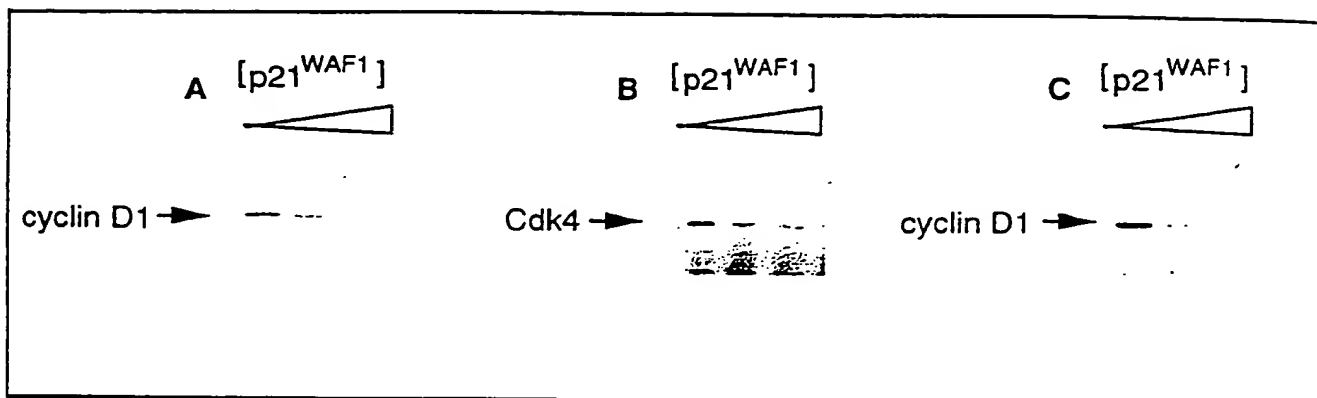
FIGURE 3



Peptide number	K_i
4 (□)	---
8 (■)	> 34.0 μM
2 (●)	2.0 μM
10 (○)	0.1 μM

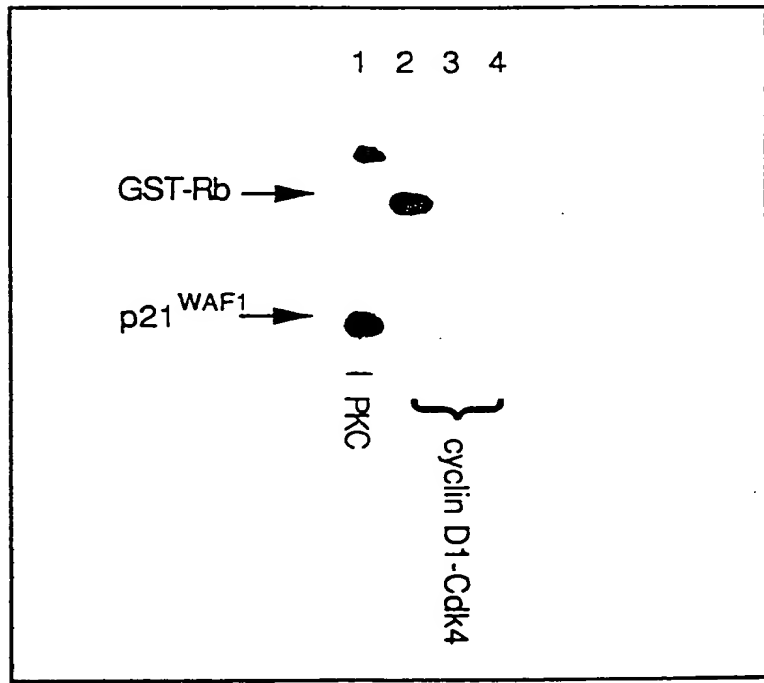
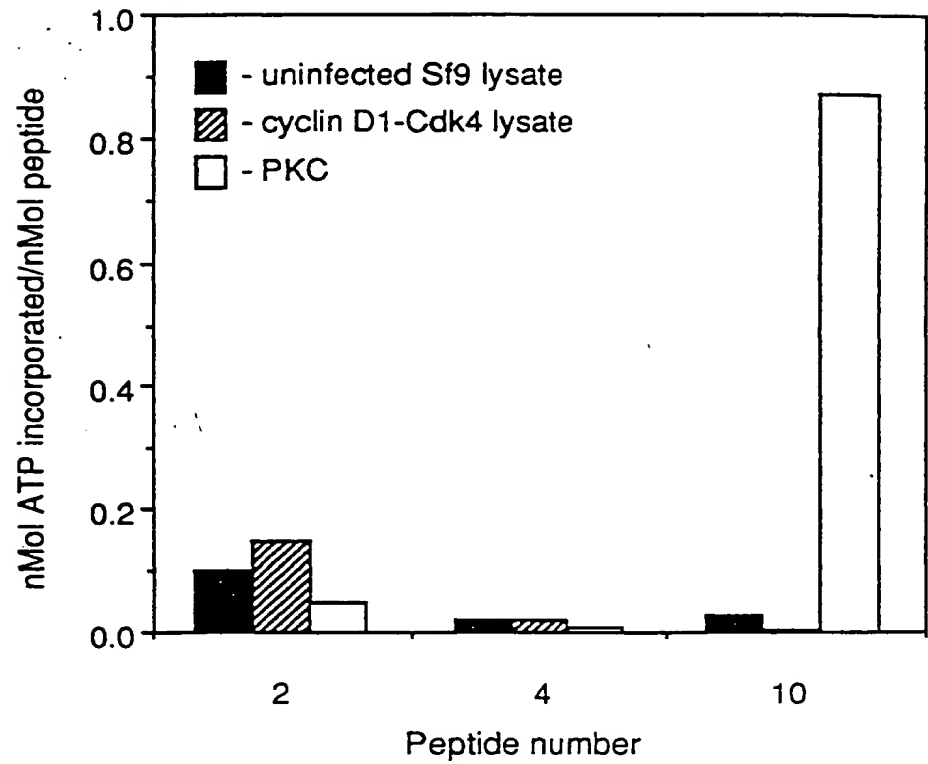
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FIGURE 4



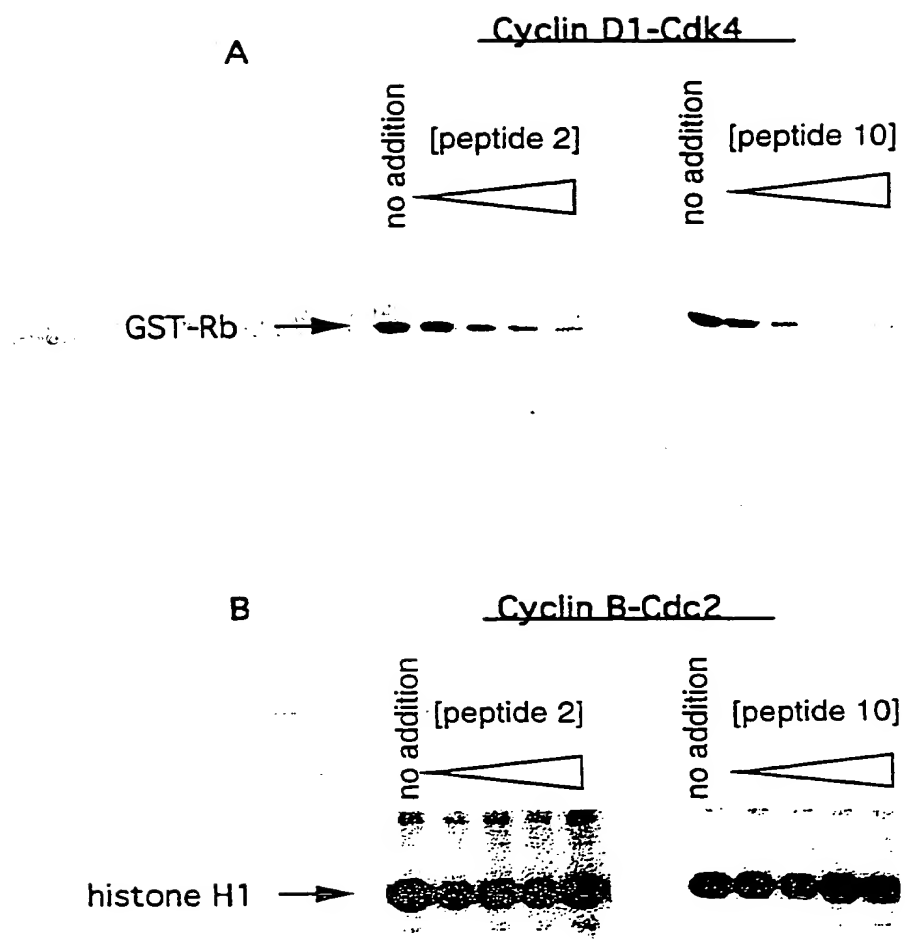
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FIGURE 5



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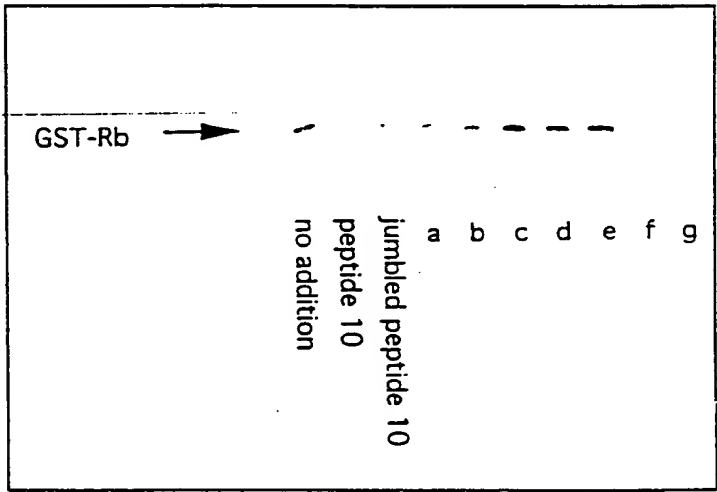
FIGURE 6



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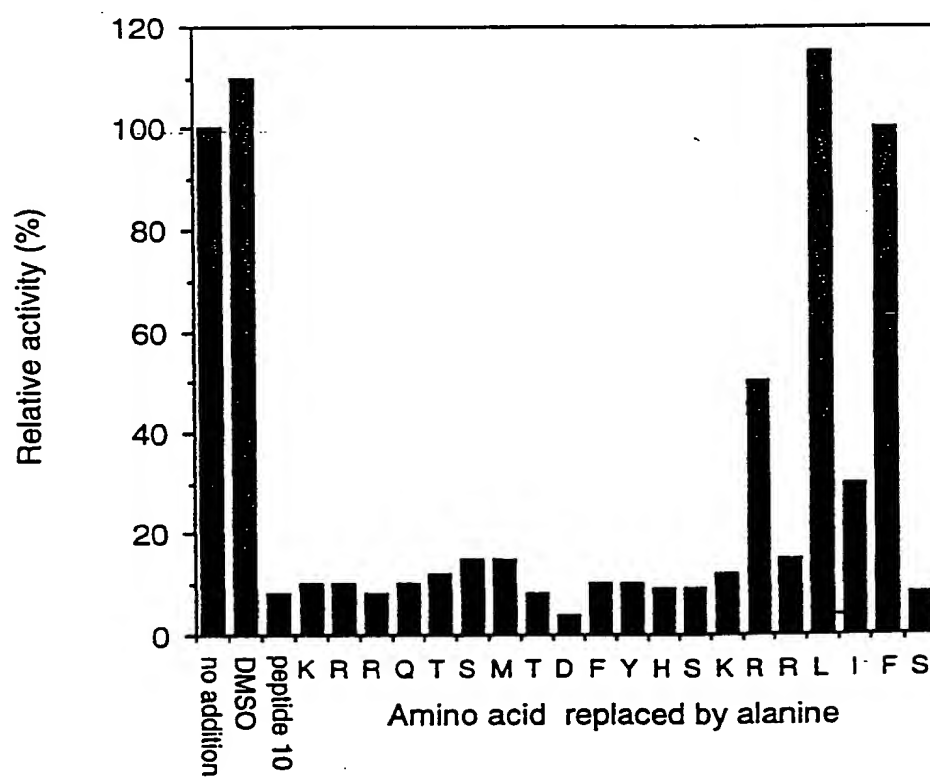
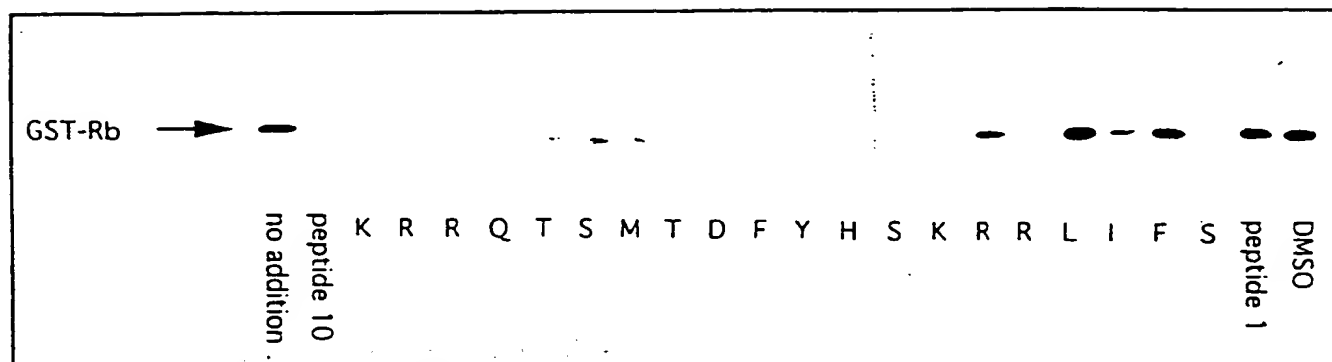
FIGURE 7

a- PRSGEQAEGSPGGPGDSQGR
b- EQAEGSPGGPGDSQGRKRRQ
c- GSPGGPGDSQGRKRRQTSMT
d- GPGDSQGRKRRQTSMTDFYH
e- SQGRKRRQTSMTDFYHSKRR
peptide 10- KRRQTSMTDFYHSKRRLIFS
f- TSMTDFYHSKRRLIFS KRKP
g- DFYHSKRRLIFS KRKP



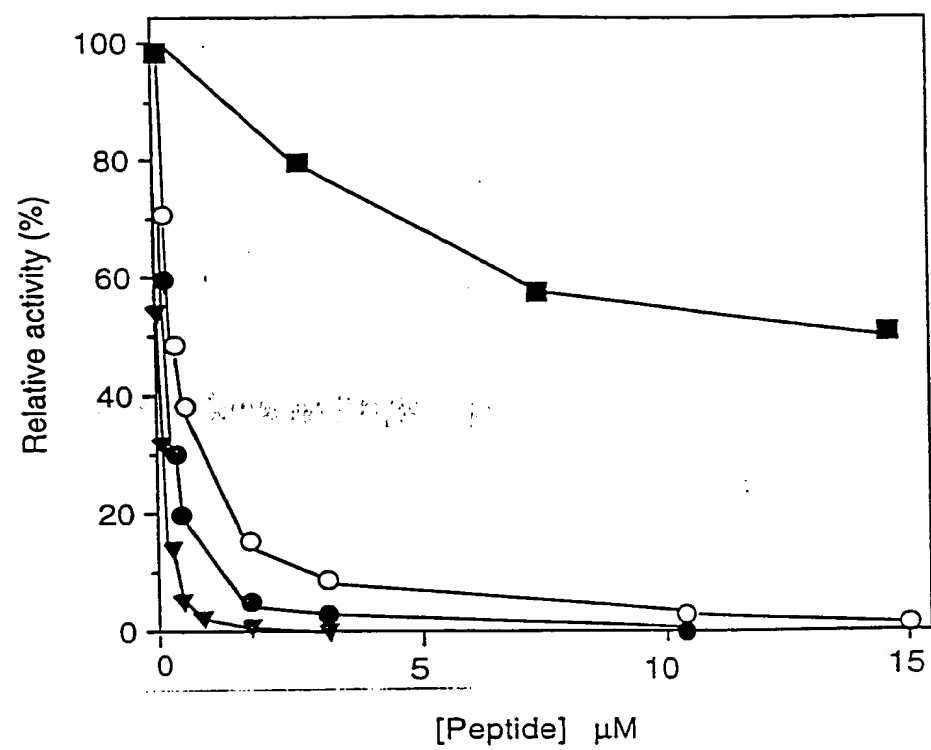
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FIGURE 8



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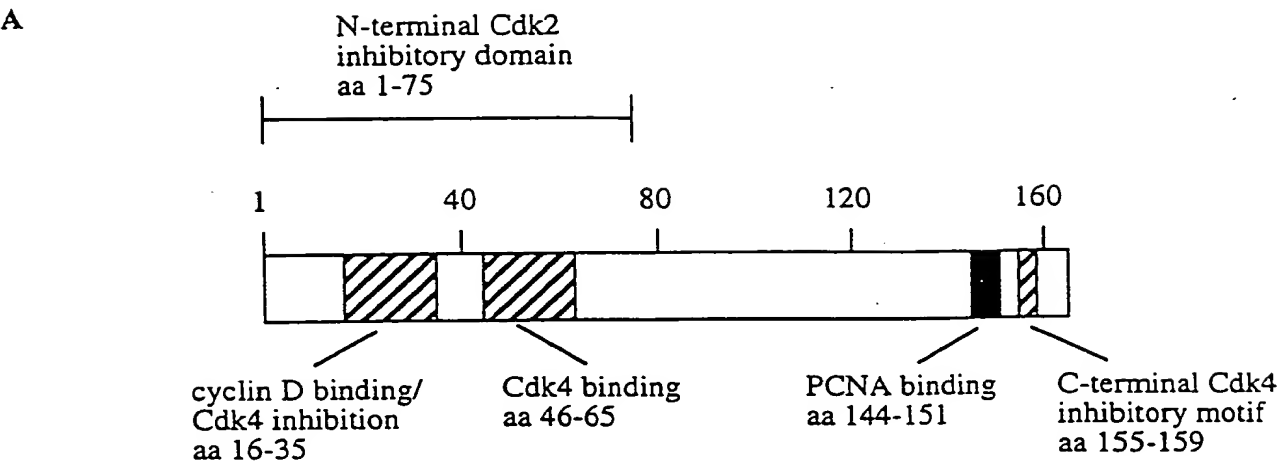
FIGURE 9



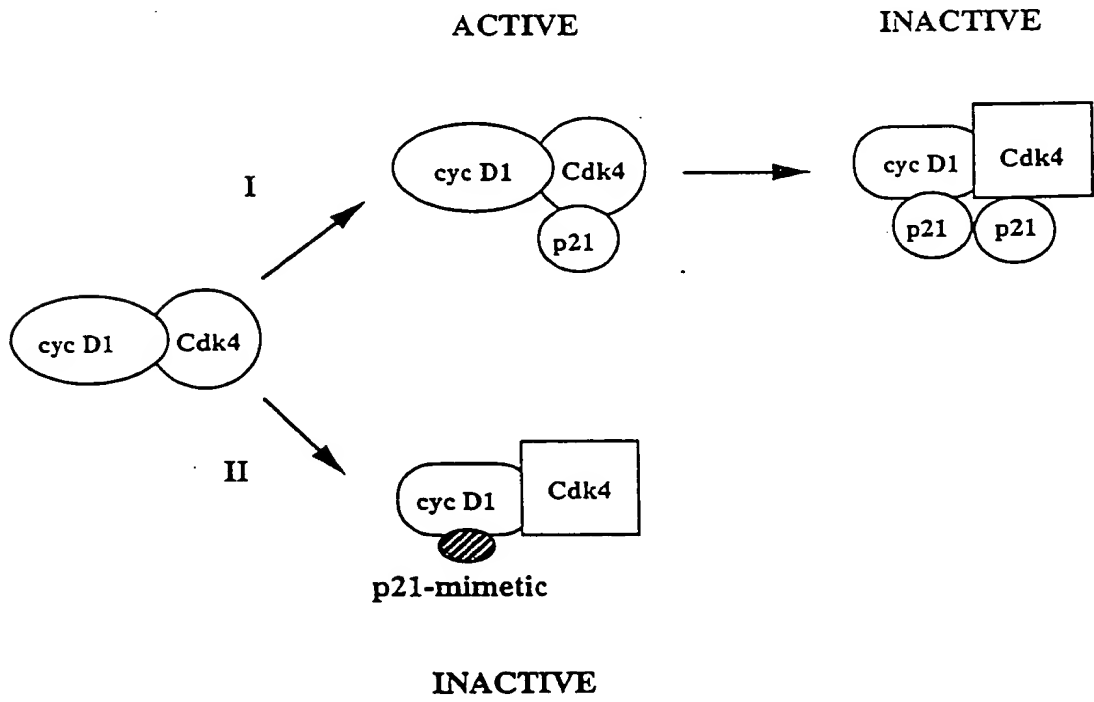
Peptide/protein	K_i
p16 ^{INK4} -peptide (■)	16.3 μ M
10 (○)	0.1 μ M
D-A mutant (○)	46 nM
full length p21 ^{WAF1} (▼)	11 nM

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FIGURE 10



B Regulation of cyclin D1-Cdk4 activity by N-terminal p21 domains



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